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UNITED STATES DEPARTMENT OF AGRICULTURE
Agricultural Research ServiceCLEANUP AND CONFIRMATION OF IDENTITY OF
PESTICIDE RESIDUES BY THIN-LAYER CHROMATOGRAPHY
PART II - CROPS AND BIOLOGICAL SAMPLESBy R. L. Schutzmann,¹ W. F. Barthel,¹ and K. A. McDonald²

In part I of this study of the impact of pesticides in agricultural environment, the use of thin-layer chromatography (TLC) for cleanup and confirmation of identity of pesticide residues in soil, water, and sediment samples was described (15).³ In part II of this study, the use of TLC for cleanup and confirmation of identity of pesticide residues in crops and biological samples is described.

Biological samples may be from mice, rats, rabbits, snakes, turtles, frogs, minnows, fish, crayfish, algae, earthworms, bees, soil insects, and other organisms selected at random from the study areas. Several authors have mentioned the potential of TLC for quantitative cleanup (4, 6, 13, 19). As with soil, water, and sediment samples, TLC offered a simple, speedy, economical method to clean up samples for gas liquid chromatography (GLC) and also to confirm identity of pesticide residues in these samples when needed (9). Previous applications of TLC to clean up samples have been reported (8, 9, 12, 14, 17).

The TLC method described here is rapid, both quantitatively and qualitatively, when used in conjunction with colorimetric, infrared, or GLC for analysis of many pesticides (9). The TLC method has a sensitivity of approximately 0.1 to 1.0 μ g. for residual perception of TLC spots when applied qualitatively. As a cleanup for instrumental methods of analysis, the capacity of the plates is as great as 100 mg. One object of this study was to compare the usefulness of TLC with other cleanup methods. The other cleanup methods tested were paper chromatography (PC) and the Florex chromatographic column (Florex). All these methods were also combined with liquid-liquid partitioning in the cleanup of samples containing excessive amounts of fats or wax.

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³ Underscored figures in parentheses refer to Literature Cited, pp. 7 and 8.

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APPARATUS

Apparatus for part II is the same as that used in part I (15) with the addition of:

Erlenmeyer flask, 500 ml., 1 liter, with 24/40 ground glass stoppers.

Graduated cylinders, 25 ml., 50 ml., and 500 ml., with ground glass stoppers.

Separatory funnels, 125 ml., 250 ml., 500 ml., and 1 liter, with Teflon stopcocks and ground glass tops.

Waring blender with vapor-proof motor.

Culture tubes, 10 ml., with Teflon-lined screw caps.

Centrifuge.

Glass tubing, 3 mm. OD, 2 mm. ID, about 8 cm. in length, with two constrictions (0.5 to 1.0 mm. ID): the first at one end of the tubing and the second at 3 to 4 cm. from that end (2).

Filter paper, Whatman, No. 3, 8 x 8 inch.

Chromatographic tank with troughs, rods, and cover (11).

Chromatographic columns, 10 x 450 mm., with a 125-ml. reservoir on top and a buret tip with Teflon stopcock on the bottom. (Kontes Cat. No. 4060.) A glass wool plug is used at the bottom.

MATERIALS

Materials are the same as those used in part I (15), except that instead of Brinkman Silica Gel G., the silica gel Adsorbosil-2 without binder was used, Applied Science Laboratories, Inc., State College, Pa.

The following materials were also used:

Florex, 60 to 100 mesh, Floridin Co., Tallahassee, Fla. Heat at 130° C. for 16 hours and store in tightly capped bottles.

Solvent--9 parts hexane and 1 part ethyl ether by volume. Solvent 2--17 parts hexane and 3 parts ethyl ether by volume.

Supercel, Johns-Manville Co., Celite Division, New Orleans, La.

Solvent, c.p., redistilled over sodium metal.

METHODS

Preparation of Plates

A layer of Adsorbosil-2 500 μ . thick was applied on 200- x 50-mm. glass plates. The plates were allowed to dry at ambient temperature overnight, then placed in a 105° to 120° C. oven for 1 hour to be activated. The plates were placed in a developing tank with redistilled ether for at least 4 hours to remove impurities that would interfere in subsequent determination by GLC. Then the plates were streaked.

Extraction

Samples Containing More Than 2 Percent Fat.

One hundred g. of finely ground sample was weighed and placed in a Waring blender jar; then 200 ml. of redistilled isopropyl alcohol (IPA) was added. The sample was homogenized for 3 to 4 minutes, then washed out of the jar with 600 ml. of redistilled pentane and transferred to a 1/2-gallon fruit jar. This jar was rotated on an extraction tumbler for 2 hours, and then the pentane

mixture was filtered through filter paper into a 1-liter separatory funnel. The IPA was removed by washing the sample three times in 400-ml. parts of distilled water (H_2O), and the interfacial cuff was discarded with the third wash. The pentane layer was drained into a 500-ml. Erlenmeyer flask and concentrated to 50 ml. on a steam bath. A 3-bulb Snyder distilling column was used. The reduced extract was filtered through a glass wool, sodium sulfate (Na_2SO_4), Celite funnel into a 50-ml. graduated cylinder, and brought to a volume of 50 ml. with pentane.

To make a Celite funnel, use a round filter tube, 150 mm. long and 35 to 40 mm. in diameter. Place a 1/2-inch layer of hexane-washed glass wool in the bottom, then a 1/2-inch layer of Na_2SO_4 , and last a 1-inch layer of Supercel brand Celite; tamp lightly and use.

Samples Containing Less Than 2 Percent Fat.

One hundred g. of ground sample was weighed and placed in a Waring blender jar; then 200 ml. of acetonitrile (CH_3CN) and 10 g. of Celite were added. All this was homogenized for 1 to 2 minutes and then decanted into two balanced 250-ml. centrifuge bottles. Each part of the sample was centrifuged and filtered through filter paper into 1-liter Erlenmeyer flasks. The pulp from each part was washed back into a Waring blender jar with another 100 ml. of CH_3CN ; and the extraction, centrifugation, and then filtration were repeated. The two CH_3CN parts were then combined and evaporated with a Snyder column. A water layer covered the bottom of the flask and water droplets appeared at the neck of the flask. One hundred ml. of hexane was added through the Snyder column to the water and then evaporated.

NOTE: A mixture of 1 volume of CH_3CN with 3 volumes of hexane produced an azeotrope boiling at 58° to 59° C., which facilitated removing the last traces of CH_3CN .

Two hundred ml. of hexane was added through the Snyder column to the water; the solution was heated to insure solution of the pesticides in the hexane, and the mixture transferred to a separatory funnel. The flask was washed with a small amount of IPA that was then added to the separatory funnel. Then the flask was washed with another 100 ml. of hexane that was added to the separatory funnel. Fifty ml. of distilled H_2O was added to remove the IPA, the solution was swirled without emulsifying, and the water layer was rejected. The hexane solution was filtered through a tube into a 500-ml. graduated cylinder, as described in the section, "Samples Containing More Than 2 Percent Fat." The separatory funnel was washed with hexane that was also filtered into the graduated cylinder and the volume was brought to 300 ml. In most cases the samples were now ready for GLC analysis.

Methods for Cleanup

For samples containing less than 2 percent fat, the following cleanup method was used:

A 5-gm. aliquot of the sample was measured into a 25-ml. graduated cylinder. If additional cleanup was required before TLC, the sample was treated with Nuchar-Attaclay mixture (15). A 0.3-ml. concentrate representing 0.5 to 5.0 g. of sample was streaked across a 200- by 50-mm. glass plate (9, 15). The amount that can be applied for each sample is shown in table 1. The benzene-hexane developing solvents used in part I of this study were also used for part II. After the plate was developed, it was allowed to stand until the solvent had evaporated. The plate was then marked to indicate the area to be examined by reference to standards. The area between marks was scraped and washed out with 5 ml. of ether into culture tubes (15). The ether was evaporated and a volume of hexane was added to give a ratio of 1 g. of sample to 1 ml. of solvent for GLC.

For samples containing more than 2 percent fat, the following cleanup methods were investigated:

(1) TLC Method: An aliquot of 0.2 to 1.0 g. of sample was streaked and the method described above for samples containing less than 2 percent fat was employed.

(2) Partitioning Method: 5 g. of sample was cleaned up by the CH₃CN-hexane method (5). A double partition was used with the following modifications:

(a) A solution of 45 ml. of CH₃CN saturated with hexane was added to a 3-g. sample in 15 ml. of hexane in a 125-ml. separatory funnel (18). The solution was shaken 1 minute and then the layers were allowed to separate. The CH₃CN layer was then drained into a 250-ml. separatory funnel.

(b) Method (a) was repeated and 30 ml. of hexane added to the 250-ml. separatory funnel. This solution was shaken 1/2 minute and then the layers were allowed to separate. The CH₃CN

Table 1.--Recovery of pesticides following cleanup by various methods

Sample material	Amount cleaned up	Amount spotted ¹	Cleanup method ²	Pesticide recovery ^{3 4}									
				Hepta-chlor	Hept. epox.	Lin-dane	Diel-drin	Endrin	P,p-DDT	O,p-DDT	P,p-DDE	Methyl para-thion	Para-thion
	Grams	Grams		Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.	Pct.
Pollen.....	5	0.5	TLC	92	88	98	100	92	100	100	79	100	103
	2.5	--	Part. and Florex	--	--	83	105	96	96	104	113	75	118
Mice.....	3	1	TLC	93	95	90	105	89	88	86	90	94	93
	5	--	Part. and Florex	--	--	--	43	24	38	34	27	74	--
Frog.....	5	1	TLC	105	83	79	100	81	86	78	75	74	79
	3	--	Florex	86	78	94	142	26	78	--	--	75	123
Bees.....	3	.5	TLC	100	98	91	100	92	100	100	100	110	113
	2.5	--	Part. and Florex	59	58	59	114	91	67	62	57	46	100
Turtles.....	5	1	TLC	86	80	89	94	88	85	85	85	105	80
	3	--	Florex	--	--	98	88	96	105	93	107	63	118
Algae.....	5	.5	TLC	73	83	74	88	75	72	71	77	130	107
	5	--	Florex	46	--	--	65	68	73	60	59	106	118
Rabbits.....	5	1	TLC	97	97	91	107	100	94	92	96	115	100
	5	--	Part. and Florex	--	40	--	59	60	64	57	60	46	90
Soybean plants.....	3	.3	TLC	71	73	68	94	100	56	63	81	116	94
	5	--	Florex	--	--	--	--	94	68	60	85	78	100
Soybean oil.....	3	1.0	Part. and TLC	66	61	64	96	79	77	51	59	--	--
Soybean oil.....	1	.1	TLC	49	--	49	57	96	55	50	50	--	--
Crayfish.....	5	.2	TLC	73	80	71	88	119	63	71	81	100	73
	3	--	Florex	--	--	--	90	150	94	85	115	29	105
Fish (minnows).....	5	.2	TLC	94	70	95	88	94	97	94	88	105	80
	3	--	Part. and Florex	83	111	86	90	58	80	74	50	46	100
Cottonseed oil.....	1	.3	TLC	99	89	88	86	103	74	82	87	68	66
	5	--	Part. and Florex	--	--	--	42	46	48	44	46	46	100
Soybean oil.....	1	.1	PC	93	94	54	100	100	56	31	29	77	35
Soybean oil.....	4	1.0	Part. and PC	67	48	45	144	76	42	30	39	--	--
Soybean oil.....	1	.2	PC	22	26	25	--	43	24	21	--	--	--
Soybean oil.....	3	.2	Part. and PC	21	25	21	48	50	30	22	31	--	--
Soybean oil.....	5	--	Part. and Florex	47	78	75	85	34	80	87	61	43	100

¹ Represents 5 mg. or less of pesticide.

² TLC refers to thin-layer chromatography cleanup; Part. refers to acetonitrile partitioning; Florex refers to Florex column cleanup; PC refers to paper chromatography cleanup.

³ By means of electron capture gas chromatography with 4-ft., 1/4-in. glass column packed with 5 percent OF-1 on Chromosorb W or 5 percent SE-30 on Chromosorb W, or both.

⁴ Average of 3 or more determinations.

layer was then drained into 300 ml. of 2 percent aqueous sodium chloride (NaCl) in a 500-ml. separatory funnel.

(c) The CH_3CN -aqueous NaCl solution was shaken for 1 minute and the funnel allowed to stand with the cap on for 15 minutes to separate the hexane layer from the rest of the solution.

(d) The CH_3CN -aqueous NaCl layer was discarded and the hexane layer filtered through anhydrous Na_2SO_4 . After the hexane layer was concentrated to 0.5 ml., a 1-g. aliquot of the sample was spotted.

(3) Partitioning-TLC Method: Methods 1 and 2 were combined.

(4) Partitioning-Paper Chromatographic (PC) Method: About 5 g. of the sample was cleaned up by the CH_3CN -hexane method (19), as described in section 2, "Partitioning Method." Depending on the amount of fat present, from 1 to 5 g. of sample was streaked across the bottom of an 8- by 8-inch filter paper and developed twice in CH_3CN (1), in a round developing tank with a glass insert and with the filter paper wrapped around the glass insert. (The filter paper must always be prewashed for a period of 8 hours in ether before use.) After it was developed and dried, the paper was turned 180° and developed with hexane back to just short of the sample streak. The paper was removed and allowed to dry. The part of the paper above the sample streak was separated and cut into small pieces. These pieces were packed into a section of constricted glass tubing (2), the paper was prewetted with benzene, and 5 ml. of the solvent was allowed to drip through the tube from a 10-ml. buret at a rate of about 5 drops per minute. The effluent was collected in a 10-ml. culture tube adjusted to a 1:1 ratio of sample to solvent for GLC determination.

(5) PC Method: From 0.2 to 1.0 g. of the sample was streaked across the bottom of a sheet of 8- by 8-inch filter paper. The PC Method, as described in section 4, "Partitioning-PC Method," was used.

(6) Florex Method: 10 g. of Florex was placed in the chromatographic column, with a 1-cm. layer of Na_2SO_4 above and a 1-cm. layer of Na_2SO_4 below the Florex. The column was tamped lightly to pack the Florex.

The column was prewashed with 50 ml. of 10 percent ether in hexane, and then 50 ml. of hexane was put on the column. The flow rate was adjusted to about 5 ml. per minute. When the level of the prewash reached the top Na_2SO_4 layer, a 10-g., or smaller, aliquot of the sample was added, and a 500-ml. Kuderna-Danish evaporator was placed under the chromatographic column. When the level of the aliquot reached the top Na_2SO_4 layer, 150 ml. of solvent 1 was added to elute the chlorinated pesticides. When this solution reached the top Na_2SO_4 layer, another Kuderna-Danish evaporator was placed under the column and 100 ml. of solvent 2 was added to elute the organophosphates, parathion and methyl parathion. The two eluates were evaporated to about 10 ml. on the steam bath with Snyder columns. After the samples were transferred to culture tubes and the volume was adjusted to give a ratio of 1 g. of sample to 1 ml. of solvent, the samples were ready for GLC determination.

(7) Partitioning-Florex Method: Methods 2 and 6 were combined.

Other methods of cleanup are not discussed in this report because they have already been reported by other investigators (5, 7, 10, 16).

RESULTS AND DISCUSSION

Adsorbents

The adsorbents were tested for three main qualities--the uniformity of the layer produced, the separation of the pesticides, and the purity of the material. Of these adsorbents, Adsorbosil-2 (without binder) was most satisfactory. The other thin-layer chromatography silica gel adsorbents

tested, and the companies that manufacture these adsorbents are as follows:

Commercial name;	<u>Source</u>
Silica Gel G (with binder) Silica Gel H (with binder)	Brinkman Instruments, Inc., Westbury, N.Y.
Adsorbosil-1 (with binder) Adsorbosil-3 (with binder)	Applied Science Laboratories, Inc., State College, Pa.
BioSil A (with binder) Biosil A (without binder)	Bio Rad Laboratories, Richmond, Calif.
Silic AR, TLC-7G (with binder)	Mallinckrodt Chemical Works St. Louis, Mo.
Woelm TLC (without binder)	Alupharm Chemicals New Orleans, La.
Chromatographic Silica Gel (with binder)	W. R. Grace and Co., Davison Chemical Division, Baltimore, Md.

Partitioning

In the partitioning method, hexane must be separated carefully from the aqueous NaCl. The separatory funnel should be kept capped to prevent the hexane from evaporating.

Extraction

For samples containing less than 2 percent fat, extracting with CH_3CN and replacing the CH_3CN with hexane is a suitable method of cleanup. This eliminates the need for a TLC cleanup.

Interferences

In most cases, as large a sample aliquot as possible had to be streaked to give a satisfactory milligram equivalent and reduce background noise on GLC. Results were best when the ratio was 1 g. or more of sample to 1 ml. of solvent. Plates had to be washed before cleanup to remove impurities remaining in the silica gel.

Plate Elution

Benzene (12) and ether both recovered pesticides from the silica gel. Both solvents, however, are highly hydroscopic. Ether was preferred because it is easily evaporated. However, ether cannot be directly injected into GLC columns. It must first be evaporated and then the residue taken up in hexane for GLC determination.

Accuracy and Precision

Although spotting techniques are quantitatively more reliable than streaking in TLC, streaking was used here because the total width of the plate could be used more effectively (9).

Loss of pesticide was minimized by using a volume of 0.3 ml. or more of solvent to apply sample aliquots to the plates.

Plate Capacity

Plate capacity can be increased either by using a larger plate or by increasing the thickness of the coating. Increasing the thickness of the coating to more than 1 mm. caused the layer of coating to crack when heated in the oven (3).

Recoveries

For samples containing less than 2 percent fat, recovery is satisfactory after cleanup of 0.3- to 1.0-g. aliquots on the TLC plates (table 1).

For samples containing more than 2 percent fat, results are best after cleanup of aliquots by CH_3CN partitioning, followed by either TLC cleanup or the Florex column cleanup, because a larger aliquot of the sample, 1 gm. or more, could be used. These two methods of cleanup gave not only a greater milligram equivalent for the subsequent GLC determination, but also a good baseline for GLC determination. Partitioning and PC cleanup, and PC, TLC, and Florex cleanups alone, for samples containing more than 2 percent fat, gave high recoveries. However, the lower milligram equivalent used in these methods gave a very poor baseline for GLC determination.

The organophosphates, parathion and methyl parathion, are oxidized rapidly in animal and plant tissues and thus values for these are not included in all the recovery studies (7).

Comparison of Methods

Using TLC for cleanup has distinct advantages.

(1) More sample weight can be spotted on TLC plates than on paper chromatograms.

(2) TLC usually gives better recovery than PC.

(3) Residues are easier to elute from TLC plates than from paper chromatograms.

For most samples with less than 2 percent fat, TLC alone is adequate. For samples with more than 2 percent fat, some fat remained, even after partitioning. When the sample was concentrated to a small volume (0.3 ml.), the fat interfered greatly with streaking and developing. PC was tried as an alternate procedure, with CH_3CN as a developing solution. The results were not much better, except those for very small aliquots of the sample (0.1 g.) (table 1). For such a small aliquot, rigorous overall cleanup is necessary to minimize background noise on GLC, as has been previously explained (p. 5). At present, partitioning, followed by column chromatography, seems to be the best cleanup method for samples with more than 2 percent fat content.

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